Localization of prostaglandin $F_{2\alpha}$ inhibition of lipoprotein use by bovine luteal cells

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Summary. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) inhibits lipoprotein-stimulated progesterone production by bovine luteal cells *in vitro* and the objective of this study was to localize the site of action of $PGF_{2\alpha}$. Cultured bovine luteal cells were treated with $PGF_{2\alpha}$ for seven days, and then with either lipoproteins or 25-hydroxycholesterol in the presence of aminoglutethimide (which inhibits cholesterol side-chain cleavage) for the final 48 h. The effects of $PGF_{2\alpha}$ on progesterone production, cellular cholesterol content, mitochondrial cholesterol content and cholesterol side-chain cleavage activity were determined. As expected, $PGF_{2\alpha}$ inhibited ($P < 0.05$) lipoprotein-stimulated progesterone production. However, $PGF_{2\alpha}$ did not inhibit low-density lipoprotein-stimulated, or high density lipoprotein-stimulated, increases in cellular cholesterol ($P < 0.05$) or inhibit lipoprotein-induced increases in mitochondrial cholesterol content ($P < 0.05$). Additionally, cholesterol content of mitochondria increased ($P < 0.05$) in the presence of $PGF_{2\alpha}$ alone. To determine if the $PGF_{2\alpha}$ -induced inhibition of steroidogenesis occurred at, or after, the side-chain cleavage reaction, we treated cells with the readily diffusable sterol, 25-hydroxycholesterol. Prostaglandin $F_{2\alpha}$ did not inhibit 25-hydroxycholesterol-stimulated progesterone production ($P < 0.05$). Prostaglandin $F_{2\alpha}$ may therefore exert its luteolytic effect at a site after cholesterol transport to the mitochondria but before cholesterol side-chain cleavage.

Keywords: corpus luteum; cow; lipoproteins; prostaglandin $F_{2\alpha}$; luteolysis

Introduction

The mechanism(s) by which prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) exerts its luteolytic effect is unknown. $PGF_{2\alpha}$ has been shown to suppress luteinizing hormone (LH)-stimulated, 3',5'-cyclic adenosine monophosphate (cAMP)-stimulated (Jordan, 1981; Pate & Condon, 1984; Benhaim *et al.*, 1987) and lipoprotein-stimulated (Pate & Nephew, 1988; Pate & Condon, 1989) progesterone production. However, $PGF_{2\alpha}$ has no effect on low-density lipoprotein (LDL) or high-density lipoprotein (HDL) uptake in bovine luteal cells (Pate & Condon, 1989), or on HDL binding (Rajkumar *et al.*, 1985) or LDL binding (Rajkumar *et al.*, 1988) in rat luteal cells, and thus $PGF_{2\alpha}$ may allow uptake of lipoprotein-carried cholesterol into the cell but inhibit the subsequent use of cholesterol for steroidogenesis.

One possible mechanism by which $PGF_{2\alpha}$ may inhibit use of cholesterol is suppression of cholesterol transport to the mitochondria, where cholesterol side-chain cleavage occurs. The cytoskeleton is involved in transport of cholesterol to the mitochondria (Hall, 1985) and a role for microfilaments in progesterone production has been demonstrated (Crivello & Jefcoate, 1978; Miller & Yin, 1978; Azhar & Menon, 1981; Gwynne & Condon, 1982; Gwynne & Strauss, 1982; Silavin *et al.*, 1984). In addition, microtubules are involved in the degradation and use of lipoproteins in rat luteal cells (Rajan & Menon, 1985). Prostaglandin $F_{2\alpha}$ may therefore disrupt the cytoskeletal network, thus preventing cholesterol transport to the mitochondria and inhibiting

cholesterol availability for side-chain cleavage. Alternatively, $\text{PGF}_{2\alpha}$ may inhibit cholesterol use by directly inhibiting the cholesterol side-chain cleavage reaction, which is the rate-limiting step in steroid synthesis and occurs via the action of the mitochondrial cytochrome P-450_{sc} enzyme complex.

The objective of this study was to localize the intracellular site of $\text{PGF}_{2\alpha}$ inhibition of the use of lipoprotein. Experiments were initially conducted to verify that $\text{PGF}_{2\alpha}$ allows lipoprotein-carried cholesterol to enter the cell. The effects of $\text{PGF}_{2\alpha}$ on cholesterol transport to the mitochondria and on the mitochondrial cytochrome P-450_{sc} reaction were then determined.

Materials and Methods

Cell culture

Mid-cycle corpora lutea (days 9–12 of the oestrous cycle, oestrus = day 0) were removed *per vaginam* from regularly cycling dairy cows of various breeds. The luteal tissue was dissociated with collagenase (2000 U g⁻¹ tissue, Type I, Worthington, Freehold, NJ, USA) and cell suspensions were placed in serum-pretreated culture flasks (25 cm², Corning Glass, Corning, NY, USA) as previously described by Pate & Condon (1982). Each experiment was repeated either six (Expts 1 and 2) or seven (Expt 3) times with different corpora lutea.

All cells were cultured in serum-free Ham's F-12 culture medium containing 24 mmol l⁻¹ Hepes (Sigma Chemical Co., St Louis, MO, USA). Culture medium was replaced with fresh medium after the initial 24 h and then every 48 h. All culture medium was supplemented with gentamicin (20 ng ml⁻¹, Grand Island Biological, Grand Island, NY, USA), penicillin (100 iu ml⁻¹), streptomycin (100 µg ml⁻¹, Pen-Strep, K. C. Biological, Lenexa, KS, USA), insulin (5 µg ml⁻¹), transferrin (5 µg ml⁻¹), selenium (5 ng ml⁻¹, ITS Premix, Collaborative Research Inc., Lexington, MA, USA) and LH (2.5 ng ml⁻¹) (USDA-bLH-B-5). This concentration of LH maintains the steroidogenic integrity of cultured luteal cells, but does not maximally stimulate progesterone synthesis. Spent medium was collected on day 7 of culture and stored at -20°C until it was analysed by radioimmunoassay. Unless otherwise indicated, all cell culture treatments were performed at the beginning of the culture period, replaced at each medium change and performed in duplicate for each corpus luteum. Control cultures did not receive any hormonal treatments. Numbers of viable cells in each flask were counted using an ocular micrometer grid with five sites counted per flask on day 6 of culture (Pate & Nephew, 1988).

Lipoprotein isolation

Lipoproteins were isolated from bovine serum obtained from regularly cycling, nonlactating dairy cattle of various breeds. LDL (density 1.006–1.063 g ml⁻¹) and HDL (density 1.063–1.21 g ml⁻¹) fractions were isolated using differential gradient ultracentrifugation using KBr for density adjustment according to the method of Havel *et al.* (1955), as modified by Radding & Steinberg (1960). The lipoprotein fractions were dialysed against a solution containing 0.15 mol NaCl l⁻¹ and 0.34 mmol EDTA l⁻¹ at pH 7.4 for 48 h; spent dialysis fluid was replaced after 24 h. The dialysed lipoproteins were sterilized by passage through a 0.22 µm Millipore filter (Millipore Corp., Bedford, MA, USA), stored at 4°C and used within four weeks of isolation. Cholesterol content in the lipoprotein fractions was determined by the method of Wybenga *et al.* (1970).

Experiment 1

Cells were cultured in the presence of aminoglutethimide (50 µg ml⁻¹, Sigma Chemical Co., St Louis, MO, USA) or $\text{PGF}_{2\alpha}$ (10 ng ml⁻¹, Sigma Chemical Co.) or both and with either LDL or HDL (25 µg cholesterol ml⁻¹). Lipoproteins and aminoglutethimide were added only on day 5 of culture. On day 7, medium was removed and cellular cholesterol was extracted with chloroform:methanol (2:1) (v/v), dried under nitrogen and stored at -20°C. The cellular cholesterol content was determined by the method of Rudel & Morris (1973). Briefly, the cholesterol was reconstituted in ethanol, treated with 33% KOH and then 95% ethanol and heated at 60°C for 15 min. After cooling, hexane and distilled water were added to each sample and thoroughly mixed. An aliquot of the hexane layer was taken, dried under nitrogen and reconstituted in an *o*-phthalaldehyde solution. Concentrated sulfuric acid was added to each sample and the absorbance read in a Beckman DU 65 spectrophotometer set at a wavelength of 550 nm.

Experiment 2

Cells were cultured in the presence of aminoglutethimide (50 µg ml⁻¹) or $\text{PGF}_{2\alpha}$ (10 ng ml⁻¹) or both and a combined LDL plus HDL lipoprotein fraction (25 µg cholesterol ml⁻¹). Lipoproteins and aminoglutethimide were added only on day 5 of culture. On day 7, mitochondria were isolated by a modification of the procedure of

Greenawalt (1974). The cells were scraped from the culture flasks into 1.5 ml microcentrifuge tubes using a rubber policeman. All steps were conducted at 0–4°C. The cells were then centrifuged at 1085 g for 5 min and the supernatant discarded. Cells were reconstituted in 300 μ l of Greenawalt's buffer, transferred to a 7 ml Dounce homogenizer and homogenized with three to four passes of each pestle. The homogenate was transferred to a 1.5 ml microcentrifuge tube and centrifuged at 755 g for 20 min; the supernatant was collected and the pellet discarded. The collected supernatant was then centrifuged at 10 800 g for 20 min to form a mitochondrial pellet and a cytoplasmic supernatant. The supernatant was retained and the mitochondria were washed once with Greenawalt's buffer. The mitochondrial pellet was then resuspended in 500 μ l of buffer. Both fractions were assayed for the cytoplasmic marker enzyme lactic dehydrogenase by the method of Bergmeyer *et al.* (1965) and the mitochondrial marker enzyme monoamine oxidase by the method of Tabor *et al.* (1954). The cytoplasmic fractions were about 72% pure, and the mitochondrial fractions were about 76% pure. Additionally, the protein in each fraction was determined using the Bio-Rad protein assay. The cholesterol in the mitochondrial pellet was extracted using chloroform:methanol (2:1) (v/v), dried under nitrogen and stored at –20°C until cholesterol determination. The mitochondrial cholesterol was derivitized by adding *N*, 0-bis (trimethylsilyl)-trifluoroacetamide and heated to 80°C for 15–20 min. Mitochondrial cholesterol content was then measured with a Hewlett-Packard 5970 mass spectrophotometer interfaced with a Hewlett-Packard 5890 gas chromatograph with a DB-1 fused silica capillary column (J & W Co., Folsom, CA, USA) using 5- α -cholestane (100 ng μ l^{–1}, Sigma Chemical Co.) as an internal standard.

Experiment 3

Cells were cultured in the presence and absence of PGF_{2a} (10 ng ml^{–1}) and 25-hydroxycholesterol (20 μ g ml^{–1}, Sigma Chemical Co.). The 25-hydroxycholesterol was administered only on day 5 of culture and the spent medium was collected on day 7 of culture for assay.

Progesterone radioimmunoassay

Progesterone concentrations in the spent culture medium collected on day 7 were determined by radioimmunoassay of unextracted samples as previously described (Pate & Condon, 1982). The intra-assay coefficient of variation was 9.6% and the interassay coefficient of variation was 18.6%. The limit of sensitivity was 0.1 ng ml^{–1}. All standards were assayed in quadruplicate and all samples were assayed in duplicate.

Statistical analysis

Within each experiment, all data were treated as a randomized complete block design with corpus luteum as the block. Differences among treatment effects were further examined using the Newman-Keuls mean separation procedure to indicate significant differences between individual treatment means. When heterogeneity of variance occurred, statistical analyses were performed on logarithmically transformed data (Zar, 1974).

Results

Cellular uptake of lipoprotein-carried cholesterol

The effects of PGF_{2a} on luteal progesterone production and on the cellular uptake of lipoprotein-carried cholesterol are summarized in Fig. 1. In Expts 1 and 3, all cells were cultured in the presence of aminogluthethimide to inhibit cholesterol metabolism for steroidogenesis and facilitate cholesterol accumulation. Similar results were obtained with cells that were not treated with aminogluthethimide (data not shown). Both LDL and HDL fractions stimulated progesterone production above control levels ($P < 0.05$). This steroidogenic response to the lipoprotein fractions was suppressed by PGF_{2a} ($P < 0.05$, Fig. 1a), although not completely reduced to control levels.

In the same cells, total cellular cholesterol was increased above controls by the addition of either LDL or HDL ($P < 0.05$, Fig. 1b). However, unlike progesterone production, PGF_{2a} had no effect on the lipoprotein-induced increase in cellular cholesterol. Cholesterol levels were increased in all lipoprotein-treated cells, regardless of PGF_{2a} treatment.

Transport of lipoprotein-derived cholesterol to the mitochondria

The potential of PGF_{2a} to inhibit cholesterol transport to the mitochondria was investigated by measuring the amount of mitochondrial cholesterol after treatment with PGF_{2a} and lipoproteins. Because similar responses were induced by both LDL and HDL, the two lipoprotein fractions were

Fig. 1. The effect of low-density lipoprotein (LDL), high-density lipoprotein (HDL) and prostaglandin F_{2α} (PGF_{2α}) on progesterone production (a) and cellular cholesterol content (b) in luteal cells is shown. Data represent day 7 of culture; PGF_{2α} treatment was continuous from day 0; LDL and HDL treatments were administered on day 5. In (a), the data were logarithmically transformed to control for heteroscedasticity. Thus, the pooled SEM from the model used to analyse the data in (a) was 171.35 ($n = 6$ separate corpora lutea). Bars in (b) represent mean $\pm$ SEM of nontransformed data ($n = 6$). Bars with different letters are significantly different from each other.

combined into a single fraction for cell treatment. The combined LDL and HDL fraction stimulated progesterone production above control values ($P < 0.05$). However, when cells were cultured in the presence of LDL, HDL and PGF_{2α}, the response to the lipoproteins was inhibited by PGF_{2α} ($P < 0.05$, Fig. 2a).

Mitochondrial cholesterol concentrations were also increased above controls by treatment with lipoproteins ($P < 0.05$, Fig. 2b). However, PGF_{2α} did not suppress the lipoprotein-induced accumulation of mitochondrial cholesterol. In fact, cells cultured in the presence of PGF_{2α} alone had higher concentrations of mitochondrial cholesterol than controls ($P < 0.05$, Fig. 2b).

Actions of PGF_{2α} on the cytochrome P-450_{SCC} enzyme complex

The ability of PGF_{2α} to act directly on the mitochondrial cytochrome P-450_{SCC} enzyme complex was investigated by treating cells with a submaximal dose of 25-hydroxycholesterol, a more readily soluble cholesterol derivative, and subsequently quantifying progesterone production. Treatment of cells with PGF_{2α} alone inhibited progesterone production to nearly half the levels of controls ($P < 0.05$, Fig. 3). Progesterone production was stimulated sevenfold above controls after treatment with 25-hydroxycholesterol ($P < 0.05$, Fig. 3). In contrast to its inhibitory effects on lipoprotein-stimulated progesterone, PGF_{2α} had no effect on 25-hydroxycholesterol-stimulated steroidogenesis.

Fig. 2. The effect of prostaglandin $F_{2\alpha}$ (PGF $_{2\alpha}$) and high-density lipoprotein (HDL) plus low-density lipoprotein (LDL) on progesterone production (a) and mitochondrial cholesterol content (b) in luteal cells. Data are from day 7 of culture; PGF $_{2\alpha}$ treatment was continuous from day 0; HDL plus LDL treatment was administered on day 5. Bars represent means $\pm$ SEM ($n = 6$ corpora lutea). Bars with different letters are significantly different.

Fig. 3. Progesterone production by luteal cells in response to 25-hydroxycholesterol (25-OHC) and prostaglandin $F_{2\alpha}$ (PGF $_{2\alpha}$). Data are from day 7 of culture; PGF $_{2\alpha}$ treatment was continuous from day 0; 25-OHC treatment was administered on day 5. Bars represent means $\pm$ SEM ($n = 7$ corpora lutea). Bars with different letters are significantly different.

Discussion

The results of Expt 1 indicate that although $\text{PGF}_{2\alpha}$ inhibits lipoprotein-stimulated progesterone production, it does not inhibit cellular uptake of cholesterol from the lipoproteins. This supports the previous suggestion that $\text{PGF}_{2\alpha}$ inhibits lipoprotein-stimulated steroidogenesis at a site subsequent to cholesterol entry into the cell (Pate & Condon, 1989). These results would also support those of Rajkumar *et al.* (1985, 1988), who found that *in vivo* administration of $\text{PGF}_{2\alpha}$ had no effect on lipoprotein binding to luteal membranes, suggesting that $\text{PGF}_{2\alpha}$ treatment does not affect the uptake of lipoprotein in rat luteal cells.

Since $\text{PGF}_{2\alpha}$ does not inhibit lipoprotein-carried cholesterol from entering the cell, it must act at some other point in the steroidogenic pathway. Several reports have suggested that $\text{PGF}_{2\alpha}$ may inhibit steroidogenesis at a site distal to cAMP production (Jordan, 1981; Pate & Condon, 1984; Kenny & Robinson, 1986; Benhaim *et al.*, 1987; Alila *et al.*, 1988; Pate & Nephew, 1988). Luteal cholesterol ester synthetase activity is inhibited and cholesterol ester stores are depleted by $\text{PGF}_{2\alpha}$ (Behrman *et al.*, 1971). This could account for the chronic inhibitory effects of $\text{PGF}_{2\alpha}$ on steroidogenesis at the time of luteolysis. However, lipoprotein-supplied cholesterol can be used directly for progesterone synthesis, bypassing storage as cholesterol ester. Thus, it is unlikely that inhibition of lipoprotein-stimulated progesterone production in this study resulted from this mechanism. An additional site of $\text{PGF}_{2\alpha}$ action may be the point of cholesterol transport to the mitochondrion or within the mitochondrion itself, where cholesterol side-chain cleavage occurs. The cytoskeleton is involved in transport of cholesterol to the mitochondrion (Hall, 1985); however, in a preliminary study in this laboratory, $\text{PGF}_{2\alpha}$ did not appear to directly affect the arrangement of the cytoskeletal elements (data not shown). In the present study, lipoproteins increased cholesterol content in the mitochondria even in the presence of $\text{PGF}_{2\alpha}$. In fact, $\text{PGF}_{2\alpha}$ alone induced an unexpected increase in mitochondrial cholesterol. Cholesterol transport to the mitochondria therefore appears not to be affected by $\text{PGF}_{2\alpha}$.

Although $\text{PGF}_{2\alpha}$ did not inhibit cholesterol transport, it did inhibit the conversion of cholesterol to progesterone. This implies that $\text{PGF}_{2\alpha}$ may inhibit progesterone production at an intramitochondrial site. However, it does not appear that $\text{PGF}_{2\alpha}$ inhibits progesterone production via inhibition of the cytochrome P-450_{SCC} enzyme complex. Treatment with 25-hydroxycholesterol caused a large increase in progesterone production, regardless of $\text{PGF}_{2\alpha}$ treatment. The 25-hydroxycholesterol readily diffuses into the mitochondria, to the site of cholesterol side-chain cleavage, and can be used as an indicator of side-chain cleavage activity (Toaff *et al.*, 1982). The increase in progesterone production in response to 25-hydroxycholesterol treatment in the present study is consistent with results obtained in rat (Rajkumar *et al.*, 1985, 1988), hamster (Silavin & Strauss, 1983) and rabbit luteal cells (McLean *et al.*, 1987), as well as in rat luteal mitochondria (Toaff *et al.*, 1982). The very large stimulation of steroidogenesis by 25-hydroxycholesterol in this study was expected since these cells had been deprived of cholesterol substrate for five days in serum-free culture. The concentration of LH used in these experiments was also not maximally stimulating, thus it is not surprising that the response to 25-hydroxycholesterol is much greater than in the presence of LH alone. It can be concluded from these results that $\text{PGF}_{2\alpha}$ does not directly inhibit the side-chain cleavage enzyme complex.

Alternatively, it is suggested that $\text{PGF}_{2\alpha}$ may inhibit progesterone production at a site subsequent to cholesterol transport to the mitochondria, but before side-chain cleavage. $\text{PGF}_{2\alpha}$ may act by an inhibition of phospholipid synthesis which is important for stimulation of cholesterol side-chain cleavage (Strauss *et al.*, 1982; Tanaka & Strauss, 1982), or inhibition of cholesterol transfer from the outer to the inner mitochondrial membrane. Further investigation is necessary to determine whether cholesterol accumulation due to $\text{PGF}_{2\alpha}$ occurs in the outer or inner mitochondrial membrane to elucidate the site(s) of $\text{PGF}_{2\alpha}$ action.

In conclusion, $\text{PGF}_{2\alpha}$ does not inhibit cellular uptake of cholesterol from lipoproteins. The luteolytic effect of $\text{PGF}_{2\alpha}$ is mediated at a site subsequent to cholesterol entry into the cell but before cholesterol side-chain cleavage, possibly at the level of the mitochondrial membranes.

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